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Lesions of the Basal Forebrain Cholinergic System Impair Task Acquisition and Abolish Cortical Plasticity Associated with Motor Skill Learning

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Summary

The contribution of the basal forebrain cholinergic system in mediating plasticity of cortical sensorimotor representations was examined in the context of normal learning. The effects of specific basal forebrain cholinergic lesions upon cortical reorganization associated with learning a skilled motor task were investigated, addressing, for the first time, the functional consequences of blocking cortical map plasticity. Results demonstrate that disrupting basal forebrain cholinergic function disrupts cortical map reorganization and impairs motor learning. Cholinergic lesions do not impair associative fear learning or overall sensorimotor function. These results support the hypothesis that the basal forebrain cholinergic system may be specifically implicated in forms of learning requiring plasticity of cortical representations.

Introduction

Plasticity associated with molecular modifications (e.g., protein phosphorylation states, insertion of receptors at synapses), synaptic modifications (e.g., long-term potentiation and depression), cellular modifications (e.g., number and shape of spines, dendrites, or axons), and system/circuit modifications (e.g., cortical representations/maps) have all been observed within the context of normal learning, suggesting that these plastic events are associated with the learned behavior. The precise contribution of each mechanism of plasticity to the many distinct forms of learning that have been described (Squire, 1992) remains to be elucidated.

One form of brain plasticity that has been documented extensively is the reorganization of adult motor and sensory cortical representations. Under a given set of circumstances, limited cortical resources are allocated in such a way that discrete subsets of cortical neurons selectively process information related to a given part of the body. Numerous studies have indicated that the amount of cortical resource allocated to a given region of the body is not fixed over time but can be altered. Plasticity of adult motor representations has been observed following either peripheral (Cohen et al., 1991; Donoghue et al., 1990; Sanes et al., 1988; Schieber and Deuel, 1997; Wu and Kaas, 1999) or central (Chen et al., 2002; Hallett, 2001; Nudo et al., 1996) lesions and following motor skill learning in intact rats, monkeys, and humans (Kleim et al., 1998; Pascual-Leone et al., 1995). The appearance of motor map reorganization under these circumstances has led to the hypothesis that this particular type of cortical plasticity may be a substrate for enabling normal motor learning and functional recovery following a lesion. To date, however, it has not been demonstrated that plasticity of cortical representations is *required* for normal learning to occur. A key reason why this hypothesis has not been examined more thoroughly is the lack of knowledge pertaining to mechanisms underlying cortical map plasticity.

The possible involvement of the basal forebrain cholinergic system in mediating cortical map plasticity underlying normal motor learning has not been directly investigated. However, prior investigations of adult sensory systems have suggested that the basal forebrain cholinergic system may play a critical role in mediating lesion-induced plasticity of sensory representations. Excitotoxic lesions of the nucleus basalis magnocellularis (NBM) in rats, the primary source of cholinergic innervation to the cortex, abolish cortical reorganization within the sensory cortex following digit amputation (Juliano et al., 1991) or peripheral nerve lesion (Webster et al., 1991). The nonspecific nature of the lesions used in these early studies prevented attributing the observed effects to the cholinergic component of basal forebrain, but nevertheless strongly suggested that the NBM-cortical projection was a likely substrate of this reorganization. More recent studies using an immunotoxin specific for cholinergic neurons within the basal forebrain have demonstrated that the basal forebrain cholinergic system is essential for mediating sensory plasticity associated with whisker pairing (Baskerville et al., 1997; Sachdev et al., 1998; Zhu and Waite, 1998). Selective blockade of cholinergic signaling within the barrel cortex similarly impairs whisker pairing plasticity (Maalouf et al., 1998). Within the auditory cortex, cortical map reorganization is enabled by nucleus basalis activity (Bakin and Weinberger, 1996; Bjordahl et al., 1998; Dimyan and Weinberger, 1999; Kilgard and Merzenich, 1998), and learning-induced plasticity is blocked, in vitro, by pharmacological blockade of cortical muscarinic receptors (Miasnikov et al., 2001). In a behavioral context, selective removal of cholinergic projections to olfactory bulb decreases a rat's ability to discriminate between perceptually similar odorants, implicating alterations in cortical representation of odorants (Linster et al., 2001). The functional/behavioral consequences of blocking cortical reorganization within sensory systems have not been thoroughly examined.

The role of the basal forebrain cholinergic system in learning has been widely debated. Early studies using nonspecific lesions of the NBM demonstrated striking deficits in a variety of learning paradigms, implicating a role for the basal forebrain cholinergic system in general learning and memory mechanisms (see review by Olton and Wenk, 1987). In contrast to these early studies, more

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recent investigations, using highly selective lesions of the cholinergic neurons in the basal forebrain, have found very modest (if any) learning deficits in many of the same behavioral paradigms studied previously with nonspecific lesions (see reviews by Baxter and Chiba, 1999; Wrenn and Wiley, 1998). In the present study, we have reexamined the role of the basal forebrain cholinergic system in learning from a different perspective. We first postulate that a key *physiological role* of the basal forebrain cholinergic system is to modulate plasticity associated with cortical representations. We then speculate that learning paradigms relying heavily upon this unique form of brain plasticity will be particularly susceptible to alterations in basal forebrain cholinergic function. To examine this hypothesis, we have investigated the effect of specific basal forebrain cholinergic lesions upon cortical reorganization associated with learning a skilled motor task and have addressed the functional/behavioral consequences of blocking cortical map reorganization. Results from this study demonstrate that disrupting basal forebrain cholinergic function completely blocks cortical map reorganization and significantly impairs, but does not abolish, acquisition of a new motor skill. Lesions of the basal forebrain cholinergic system do not lead to a deficit in associative fear learning or impair general sensorimotor function. These results support the hypothesis that the basal forebrain cholinergic system may be specifically implicated in forms of learning that require plasticity of cortical representations.

Results

A total of 64 adult male F344 rats were used in this study and were assigned to one of three basic experimental paradigms. (1) To initially establish the behavioral consequences of lesions of basal forebrain cholinergic neuronal projections to the cortex, 11 animals received bilateral lesions of the NBM prior to forelimb reach training, and 17 unlesioned animals served as controls. (2) To determine whether basal forebrain cholinergic lesions affect retention of a previously learned skilled motor behavior, additional rats were first trained to perform the skilled forelimb reaching task, and then received either bilateral lesions of the NBM cholinergic cell group ($n = 12$), bilateral injections of vehicle ($n = 3$), or sham surgeries ($n = 9$). (3) A third set of animals was used to investigate the effect of combined lesions of the NBM and medial septum cholinergic systems. For this purpose, six animals received bilateral immunotoxic lesions of cholinergic neurons in both the medial septum and NBM, three animals received comparable injections of vehicle, and three animals remained intact. Following forelimb reach training, this third group of animals underwent electrophysiological mapping to determine the caudal forelimb representation within the motor cortex and were evaluated for possible deficits in associative fear learning and general sensorimotor function. Throughout the study, behavioral training always consisted of 15 days of forelimb reach training, 60 trials per day, with the first 3–4 days devoted to shaping the behavior. In all cases, a period of 10–14 days was permitted between any surgical manipulation and behavioral testing to permit sufficient recovery from the surgical procedure and to allow the immunotoxin sufficient time to fully deplete basal forebrain cholinergic function.

As described above, an initial study was carried out to determine whether lesioning the basal forebrain cholinergic system would impair learning of a skilled motor task. For this purpose, one group of animals ($n = 11$) received bilateral lesions of the cholinergic neurons of the NBM. A second group of rats remained intact ($n = 17$). Following a 2 week postsurgical interval, rats were trained on a skilled motor task that involves learning to use the forepaw to retrieve small food pellets from a platform adjacent a test chamber (Whishaw, 2000). By test day 5, all rats were capable of completing 60 training trials within a 10 min period. Both groups of rats showed significant improvement in reaching performance over the training period (Figure 1). However, nonlesioned rats learned the task more rapidly and achieved an overall higher level of accuracy than did SAP-lesioned animals (average accuracy across all trials = 21.2% $\pm$ 4.1% in NBM-SAP-lesioned animals compared to 47.4% $\pm$ 3.7% in intact animals; $t(26) = 4.65$; $p < 0.0001$ unpaired t test). A post hoc analysis by day indicated that intact animals performed significantly better than SAP-lesioned animals on all but the first testing day.

A histological evaluation indicated that SAP lesions eliminated nearly all cholinergic neurons within the NBM and substantia inominata and dramatically reduced cholinergic innervation within the motor cortex (Figure 2). A quantitative analysis indicated that SAP lesions reduced cholinergic innervation to the cortex by an average of more than 99% (Table 1) and the cholinergic lesion exceeded 98% completeness in every subject. Thionin-stained sections indicated that a distinct needle tract could be seen in all SAP- and vehicle-injected animals, but there was no additional nonspecific tissue damage. Parvalbumin-stained sections taken through the NBM indicated that SAP lesions specifically depleted cholinergic neurons but did not result in noticeable damage to the GABAergic cells within this region (Figures 2E and 2F).

To determine whether residual learning in NBM-lesioned animals may have been associated with unlesioned basal forebrain cholinergic neurons arising from the medial septum (MS), which primarily innervate the hippocampus and cingulate cortex, an additional experiment was carried out. For this study, one group of animals ($n = 6$) received bilateral lesions of both the NBM and the MS, while control animals either received injections of vehicle solution in identical locations ($n = 3$) or remained intact ($n = 3$). All animals were then trained to acquire the skilled motor task as in the preceding experiment. Animals with combined lesions of the MS and NBM were significantly impaired in their ability to learn the motor task relative to either intact or vehicle-injected animals [overall repeated measures ANOVA $F(2,9) = 9.87$; $p = 0.005$; post hoc Fisher's comparing SAP-lesioned versus vehicle injected, $p = 0.007$; SAP-lesioned versus intact, $p = 0.004$]. Vehicle-injected and intact animals did not differ in performance ($p = 0.77$ post hoc Fisher's) and were therefore combined into a single group for further analysis. When data from animals in all experiments were combined (23 controls [vehicle + intact]; 11 NBM-alone lesions; 6 MS + NBM

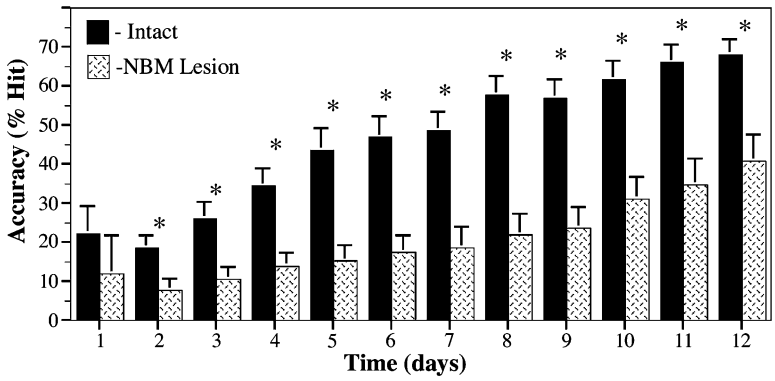

Figure 1. Selective Lesions of Cholinergic Neurons in the NBM Significantly Impair Acquisition of a Skilled Motor Task

Animals with bilateral immunotoxic lesions of the NBM (hatched; n = 11) were significantly impaired in learning a skilled motor task when compared to intact (black; n = 17) rats. While both groups of rats improved in reaching accuracy over the training period [$F(1,26) = 22.8$; $p < 0.0001$; repeated measures ANOVA for time], intact rats learned more rapidly [$F(10,260) = 3.26$; $p < 0.0005$; repeated measures ANOVA treatment × time] and achieved an overall higher level of accuracy. A post hoc analysis indicated that intact rats performed better than SAP-lesioned ones on all but the first day of testing (*$p < 0.05$ days 2, 3; $p < 0.005$ days 4, 5, 12; and $p < 0.0005$ days 6–11; unpaired t test). All rats performed 60 reaches per day, except on test day 1, when only five intact and five SAP-lesioned rats performed reaches, and these animals only completed 50 reach trials each.

lesions) and statistically examined, it was apparent that lesions of the cholinergic system significantly impaired motor learning. Animals with bilateral lesions of the NBM, or with combined bilateral lesions of the NBM + MS, were significantly impaired in learning relative to intact animals [overall repeated measures ANOVA treatment × time, $F(20,370) = 2.05$; $p = 0.005$; post hoc Fisher for NBM and combined NBM + MS lesions versus control animals, $p < 0.001$]. Animals with combined lesions of the MS + NBM acquired the task to the same extent as animals with NBM lesions only (post hoc Fisher's, $p = 0.43$). Thus, the observed effects of basal forebrain cholinergic lesions on learning performance were attributable to a deficit in the NBM-cortical cholinergic system.

The initial results clearly indicated that selective depletion of cholinergic inputs to the cortex significantly impaired an animal's ability to learn a skilled motor task. To determine whether the basal forebrain cholinergic system was required only for acquiring a skilled motor

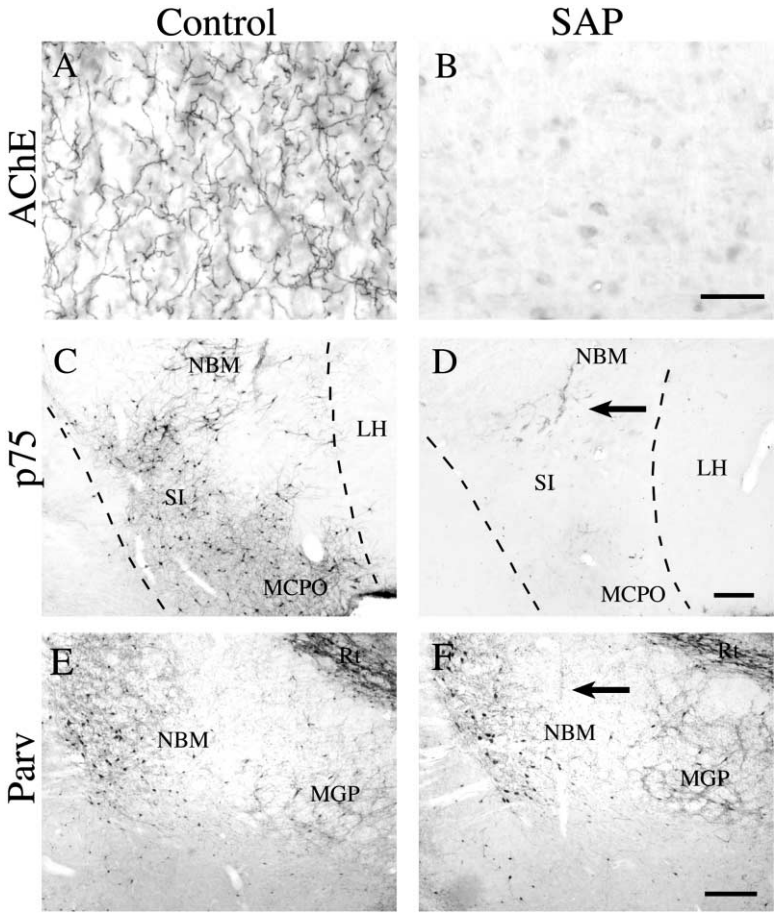

Figure 2. 192-IgC Saporin Lesions Deplete Cholinergic Neurons and Their Axons

Intraparenchymal injections of 192-IgG saporin (SAP) into the NBM selectively destroy cholinergic cell bodies within the NBM and eliminate cholinergic afferent innervation to the cortex. Cholinergic fibers labeled for AChE were found throughout the cortex, including the primary motor cortex (A). Cholinergic fibers were rarely seen in the cortex of animals with bilateral injections of SAP into the NBM (B). Cholinergic cell bodies, expressing the p75 receptor, are normally scattered throughout the basal forebrain, including the NBM and substantia inominata (C). Intraparenchymal injections of SAP within the NBM eliminated virtually all p75-expressing cells in the NBM and substantia inominata (D) but did not affect cells within the medial septum (not shown). The arrow in (D) indicates the most rostral injection site (a second injection was placed 1.2 mm caudal and 1.5 mm lateral to the first site). Sections stained for parvalbumin (E and F) demonstrated that SAP lesions did not damage GABAergic cells within the basal forebrain. Similar numbers of parvalbumin-labeled cells were noted in control (E) and SAP-lesioned (F) animals. Scale bar in (B) equals 50 μm and applies to (A) and (B). Scale bar in (D) equals 250 μm and applies to (C) and (D). Scale bar in (F) equals 250 μm and applies to (E) and (F).

Table 1. SAP Lesions Resulted in >99% Loss in Basal Forebrain Cholinergic Innervation to the Sensorimotor Cortex

	AChE-Positive Fiber Crossings/mm^2		
	Control (n = 6)	SAP (n = 6)	% Loss
Layer II-III	83.89 ± 9.47	0.56 ± 0.22	99.33% ± 0.267%
Layer V-VI	194.2 ± 19.4	1.32 ± 0.55	99.32% ± 0.284%

Unbiased techniques were used to randomly sample sites within layers II-III and layers V-VI throughout the rostral-caudal extent of the sensorimotor cortex. Columns 2 and 3 indicate the average number of AChE-positive fibers crossing the counting frame per animal. Column 4 indicates the average loss in cholinergic fiber density following SAP lesions of the NBM.

behavior, or was necessary for executing a previously learned behavior, additional experiments were performed. To determine the effects of selective cholinergic lesions on performance of a previously acquired skilled motor task, 24 rats were trained in the skilled forepaw task prior to lesioning the cholinergic cells of the NBM. All rats acquired the task to a comparable level and were subsequently divided into three experimental groups for lesioning: 12 rats received bilateral SAP lesions of the NBM, 3 received vehicle injections within the NBM, and 9 were left unoperated. Following a 10 day recovery period, rats underwent an additional week of behavioral testing. An analysis comparing the performance of each animal during the week prior to lesioning and the week following lesioning indicated that lesions of the basal forebrain cholinergic system do not affect performance of a previously learned skilled motor behavior (Figure 3). These data clearly demonstrate that, although the basal forebrain cholinergic input to the cortex is critical for enabling an animal to learn a new skilled motor behavior, this system is not required for an animal to perform a previously acquired motor task. Moreover, these data demonstrate that basal forebrain cholinergic lesions alone do not impair motor function in the skilled forelimb task, strongly suggesting that the functional deficit seen in the acquisition phase of testing is due to a learning deficit rather than a motor impairment.

The physiological basis for the learning deficit in animals lacking cholinergic innervation to the cortex could not be determined from the behavioral studies. Previous studies, however, have indicated that skilled motor learning tasks, similar to those used in the present study, lead to the selective expansion of the caudal forelimb representation within the motor cortex (Kleim et al., 1998; VandenBerg et al., 2002). The expansion of motor representations has been observed in rats, monkeys, and humans following motor skill learning, suggesting that cortical map plasticity was a substrate for the learning.

To test the hypothesis that basal forebrain cholinergic lesions may impair skilled motor learning by disrupting plasticity of cortical representations, an additional study was performed using functional mapping techniques to derive cortical representations within the primary motor cortex. For this study, 12 trained animals (6 combined MS + NBM-SAP-lesioned, 3 vehicle, and 3 intact from the above behavioral study) and 6 untrained rats were used. The functional motor representation of the caudal forelimb area was determined using electrical stimulation techniques. In all cases, movements elicited by contraction of the shoulder muscles were kept distinct from forepaw movements and were not included when calculating the total area of the caudal forelimb representation. The size of forelimb representations from vehicle and intact animals was not statistically different ($p = 0.51$ trained side and $p = 0.54$ untrained side post hoc Fisher's analysis comparing Veh and intact groups), and the two groups were combined for additional analyses.

Figure 4 illustrates representative motor maps obtained from the cortex contralateral to the trained hand (all rats had a preferred paw that was used throughout the training period) in control and SAP-lesioned trained animals and from untrained animals. Since untrained animals did not have a "preferred paw," the size of the forepaw representation obtained from both hemispheres was averaged. As seen in the representative maps and confirmed in a statistical analysis of all experimental animals, our results indicate that skilled motor

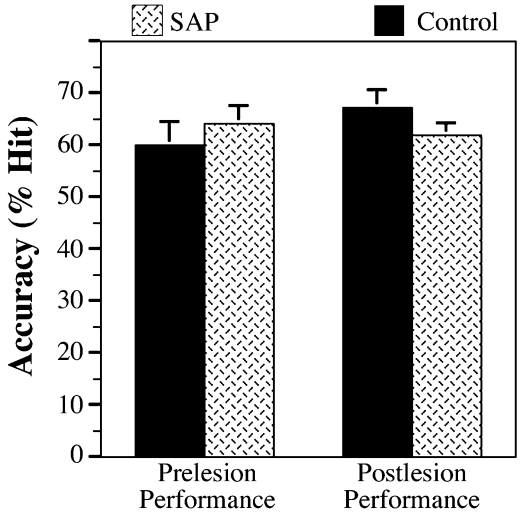

Figure 3. Lesions of the Basal Forebrain Cholinergic System Do Not Impair Performance of a Previously Acquired Skilled Motor Behavior

All rats (n = 24) were initially trained for 3 weeks to acquire the forelimb reach task. As expected, there was no difference between the groups of animals in reaching accuracy during the week prior to the lesion ("Prelesion"; average of last 5 days of acquisition training; 60 trials per day; $t(22) = 0.721$; $p = 0.48$ unpaired t test). After learning the task, 12 of the rats were given bilateral immunotoxic lesions of the NBM cholinergic neurons. After a 2 week postsurgical recovery, all rats underwent an additional week of behavioral testing (5 days; 60 trials per day). During the "postlesion" period, no differences in reaching accuracy were found between SAP-lesioned (n = 12) and control (n = 3 vehicle + 9 intact) rats ($t(22) = 1.29$; $p = 0.21$, unpaired t test). A paired t test comparing the performance of SAP-lesioned animals before and after the lesion indicated no significant loss of reaching accuracy occurred as a result of the lesion ($t(11) = 0.64$; $p = 0.53$).

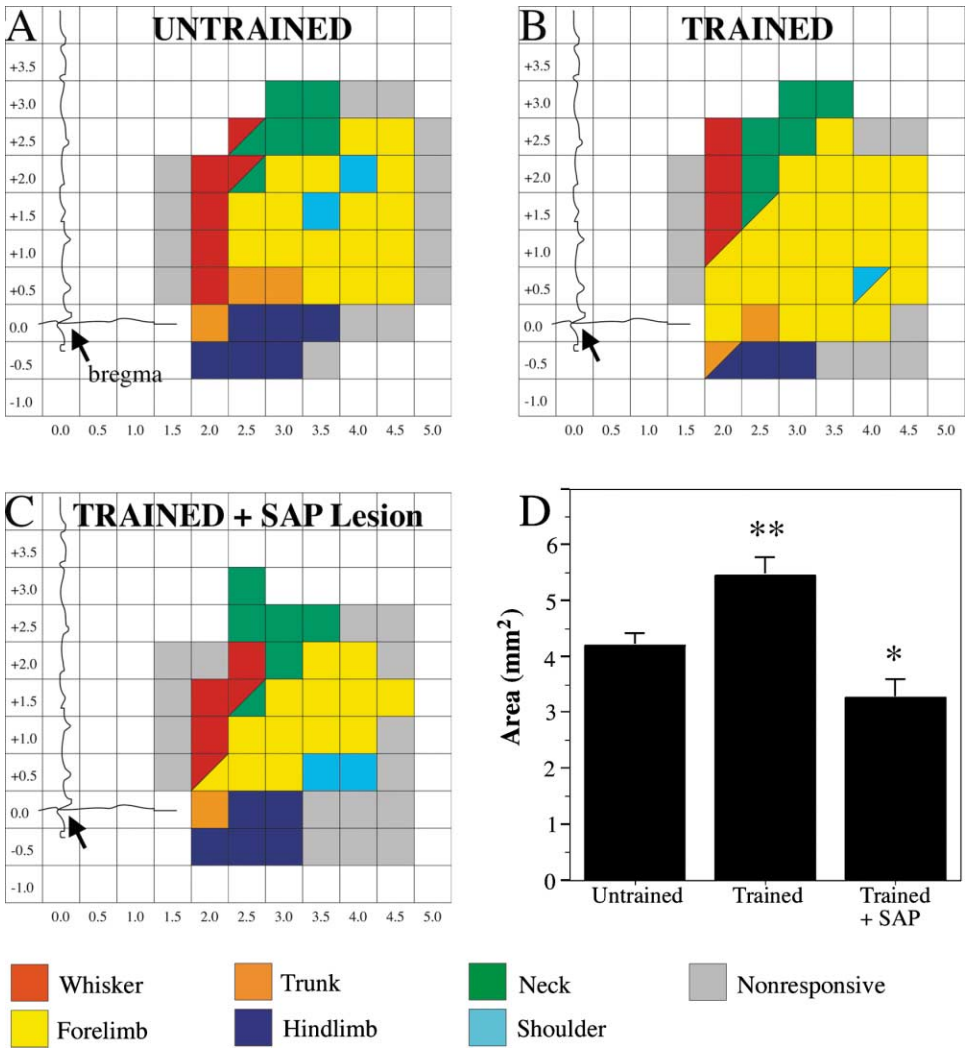

Figure 4. Lesions of the Basal Forebrain Cholinergic System Block the Expansion of the Forelimb Representation Induced by Reach Training

Motor maps were generated using conventional electrical stimulation techniques. Panels (A)–(C) show representative motor maps from untrained (A), trained/control (B), and trained/SAP-lesioned (C) rats. Numerical values on the *x* and *y* axes represent medial/lateral and anterior/posterior coordinates, respectively, measured (in millimeters) from Bregma (indicated by the arrow). Maps in (B) and (C) were obtained from the cortex contralateral to the trained paw. The size of the caudal forelimb representation (indicated in yellow) is increased in trained animals (B) relative to untrained controls. In animals with basal forebrain cholinergic lesions (C), the training-induced expansion of the forelimb representation is abolished. Panel (D) illustrates the average size of the forelimb representation obtained from cortex controlling the trained paw in untrained (average of both hemispheres since no preferred paw was determined; n = 6), trained/control (n = 6), and trained/SAP-lesioned (n = 6) animals. A significant group affect was observed [$F(2,15) = 13.80$; $p = 0.0004$ overall ANOVA]. Moreover, training induced a significant expansion in the size of the caudal forelimb representation relative to untrained animals ($p = 0.009$, post hoc Fisher's). Lesions of the basal forebrain cholinergic system completely blocked the training-induced expansion of the forelimb (**$p = 0.001$, post hoc Fisher's comparing control and SAP-lesioned animals) and significantly reduced the forelimb representation relative to untrained control animals (*$p = 0.042$, post hoc Fisher's).

learning is associated with a significant expansion of the caudal forelimb representation for the trained limb (30.3% ± 7.7% increase in trained animals compared to untrained; $p = .009$ post hoc Fisher's). More importantly, these data indicate that the expected expansion is completely blocked in animals with selective lesions of the basal forebrain cholinergic system. Moreover, a 22.4% ± 8.0% decrease was seen in the size of the forelimb representation in SAP-lesioned animals compared to untrained control animals ($p = 0.04$ post hoc Fisher's comparing SAP and untrained animals). An analysis of the cortical forepaw representation was also carried out for cortex controlling the untrained hand (not shown in Figure 4). These data indicated significant group differences in the size of the caudal forepaw representation of the untrained hemisphere [ANOVA $F(2,15) = 9.08$; $p = 0.0026$]. A smaller, but significant, 24.3% ± 10.7% increase ($p = 0.04$ Fisher's post hoc analysis between trained controls and caged animals) occurred in the untrained forepaw representation of trained animals relative to caged controls. A 22.4% ± 6.2% decrease in the size of the forepaw representation in the untrained hemisphere was again seen in the SAP-lesioned animals compared to caged controls, although

this difference did not quite achieve significance ($p = 0.06$, post hoc Fisher's comparing SAP-lesioned and caged animals). An analysis of the cortical representations from the trained and untrained forepaw in unlesioned animals (vehicle and intact combined) indicated that a comparable increase in the size of the forepaw representation occurred in both hemispheres ($t(5) = 0.463$; $p = 0.66$ paired t test for intact and vehicle animals).

To determine whether differences in stimulation parameters may have influenced motor map plasticity among the treatment groups, the minimum and average stimulus intensity required to elicit an evoked movement of the forelimb was determined for each animal. No significant group differences were seen in either minimum or average stimulus intensities required to evoke a forelimb movement for either the trained or untrained hemispheres [minimum threshold/trained, $F(2,15) = 1.99$; $p = 0.17$; minimum threshold/untrained, $F(2,15) = 2.1$; $p = 0.16$; average threshold/trained, $F(2,15) = 1.99$; $p = 0.17$; average threshold/untrained, $F(2,15) = 2.1$; $p = 0.14$].

Results from the present study clearly demonstrate that impairing basal forebrain cholinergic function disrupts cortical map reorganization and significantly impairs acquisition of a new motor skill. These data support the hypothesis that the basal forebrain cholinergic system may be an essential contributor to mechanisms leading to shifts in cortical motor representations, a form of plasticity that is required for skilled motor learning to take place. To further support this hypothesis, additional experiments or analysis were conducted to exclude the possibility that motor learning impairments observed following basal forebrain cholinergic lesions were the result of either (1) attention deficits, (2) more generalized learning impairments, or (3) nonspecific sensorimotor deficits.

Prior studies have strongly suggested that the basal forebrain cholinergic system, particularly the NBM-cortical system, influences attention (Chiba et al., 1995, 1999; McGaughy et al., 2002; McGaughy and Sarter, 1998; Sarter et al., 2001; Wenk, 1997). Given this previous experimental evidence, we sought to determine whether the learning deficit seen in the SAP-lesioned animals could be accounted for simply because their attention was not engaged by the task. To assess the extent to which each animal maintained vigilance across the behavioral task, the time to perform the required 60 trials was recorded each day. Each animal performed the required 60 trials on every testing day, and the time required to complete each day's trials was not different when comparing SAP-lesioned and control animals across the testing period [Figure 5; repeated measures ANOVA $F(11,110) = 1.21$; $p = 0.29$ for treatment $\times$ time; and a post hoc analysis using a t test to compare times for each day indicated SAP-lesioned animals only took significantly longer to complete the trials on one testing day, day 14]. These data suggest that all animals maintained attentional set and that global attention deficits were not responsible for the impairment in motor learning observed in this study, although the contribution of more subtle deficits in attention cannot be ruled out.

To determine whether lesions of the basal forebrain cholinergic system were specifically impairing motor learning, we also examine the role of the corticopetal cholinergic system in an associative fear learning task. Fear conditioning is a robust, rapidly acquired form of associative learning, which relies primarily on separate brain structures and different memory systems than the skilled motor task (Goosens and Maren, 2001; LeDoux, 2000; Maren, 2001). Fear conditioning consisted of a single training session including ten tone-shock paired presentations, followed by a 10 day consolidation period and a single testing session including ten tone-only presentations. In rodents, decreased activity is an expression of learned fear (LeDoux, 2000). The degree of learning for each group is expressed as a percentage of the activity rate during the tone presentations relative to the activity rate during the baseline period. No differences were observed between the SAP-lesioned and control animals in the degree of learning with fear conditioning (Figure 6). This result supports the hypothesis that basal forebrain lesions do not impart global associative learning deficits and suggests that the impairment in motor learning cannot be accounted for by a general learning deficit, but rather that it is specific to a cortical motor memory system. These data are consistent with the finding that lesions of the cholinergic neurons in the nucleus basalis of rats fail to impair aversive learning in an inhibitory avoidance task, whereas the lesions appear to block memory enhancement by posttraining norepinephrine in the basolateral amygdala (Power et al., 2002).

As an additional control, we investigated whether impairments in motor learning following lesions of the basal forebrain cholinergic system were simply due to overall impairments in sensorimotor function rather than a learning deficit per se. For this purpose, animals were evaluated on a horizontal ladder task that requires rats to cross an 8 foot span of evenly spaced wire rungs. This task has been used previously to identify sensorimotor deficits following acute brain trauma (Metz and Whishaw, 2002) or spinal cord injury (Miya et al., 1997; Soblosky et al., 2001). Forelimb and hindlimb footfalls were counted over a series of seven trials conducted over two days. SAP-lesioned animals did not differ from control animals in their performance on the ladder task (Figure 7), crossing with few, if any, forelimb footfalls. These data indicate that the motor learning impairment seen in animals with basal forebrain cholinergic lesions is not associated with nonspecific defects in sensorimotor function but is more likely a reflection of deficits in motor learning.

Discussion

Data from the present study provide the first experimental evidence demonstrating that the basal forebrain cholinergic system is *essential* for mediating cortical plasticity associated with normal learning and support the hypothesis that cortical map reorganization is a key substrate for enabling an animal to effectively learn a skilled motor behavior. The present study demonstrates that acquisition of a skilled motor behavior is normally associated with an expansion of cortical representation of the trained limb and further demonstrates that the expansion occurs bilaterally in trained animals. Selective lesions of the basal forebrain cholinergic system

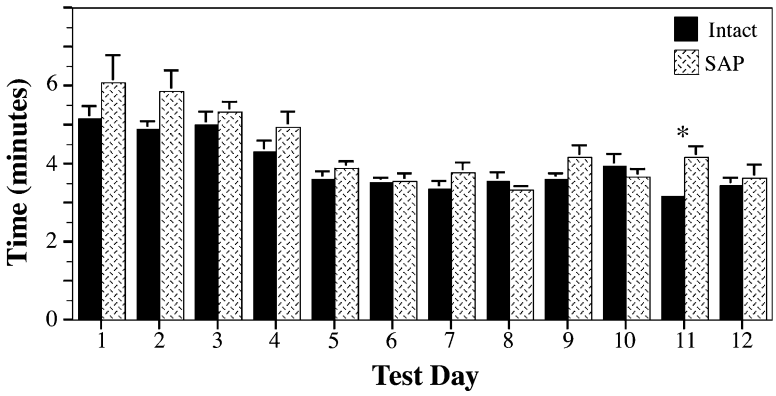

Figure 5. Lesions of the Basal Forebrain Cholinergic System Did Not Impair the Animals' Ability to Attend to the Forelimb Reach Task

To assess the extent to which each animal attended to the behavioral task, the time to perform the required 60 trials was recorded each day. While all animals demonstrated a significant reduction in the time to perform the task over the course of training [$F(11,110) = 16.56$; $p < 0.0001$ repeated measures ANOVA for time], there was no difference between SAP-lesioned and control rats [$F(11,110) = 1.22$; $p = 0.29$ repeated measures ANOVA treatment $\times$ time].

disrupt the training-induced cortical reorganization in both hemispheres and impair, but do not abolish, the animal's ability to learn a skilled motor behavior.

The hypothesis that cortical reorganization contributes to learning a new motor skill has thus far been supported only by correlative data demonstrating that the plasticity occurs when learning has taken place (Classen et al., 1998; Karni et al., 1995; Kleim et al., 1998; Pascual-Leone et al., 1995; Plautz et al., 2000; Sanes and Donoghue, 2000). The present study adds additional critical support for this hypothesis by demonstrating that, in the absence of the cortical plasticity, motor skill learning is significantly impaired. These data either indicate that the cortical plasticity is directly responsible for the learning or that both phenomena are associated with a common cortical process that requires basal forebrain cholinergic modulation. Importantly, prior studies have indicated that the induction of cortical plasticity within auditory cortex by intracortical microstimulation does not, by itself, induce learning (Talwar and Gerstein, 2001), suggesting that cortical reorganization may be necessary but not sufficient to acquire a new behavior.

The critical role of the basal forebrain cholinergic system for enabling the reorganization of cortical representations during normal motor learning is clearly demonstrated in the present study. Prior studies examining plasticity of sensory representations have demonstrated that nonselective excitotoxic lesions of the nucleus basalis completely abolished cortical map reorganization following digit amputation (Juliano et al., 1991) or nerve transection (Webster et al., 1991). Cortical plasticity associated with whisker pairing (a form of sensory deprivation) is also completely abolished by selective lesions of the basal forebrain cholinergic system (Baskerville et al., 1997; Sachdev et al., 1998; Zhu and Waite, 1998). Taken together, these data indicate that the basal forebrain cholinergic system plays a critical role in mediating

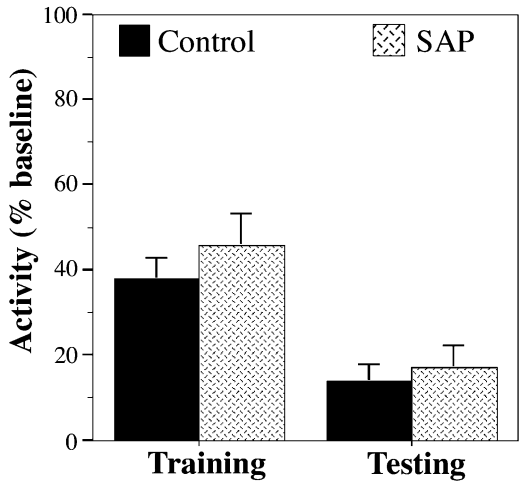

Figure 6. Lesions of the Basal Forebrain Cholinergic System Do Not Impair Animals in a Fear-Conditioning Paradigm

The degree of learning in this paradigm is expressed as the percent activity during the tone relative to the baseline activity rate (lower activity indicates stronger learning). Control and SAP-lesioned (NBM + MS) animals exhibited significant reductions in spontaneous activity during both the training and testing phase of the paradigm. However, there was no difference in the degree of learning between groups during the training phase (control = 37.7 ± 5.0, SAP = 45.7 ± 7.5) ($t(10) = 0.53$; $p = 0.61$, unpaired t test) or the testing phase (control = 13.8 ± 3.8, SAP = 17.1 ± 4.9) ($t(10) = 0.885$; $p = 0.40$, unpaired t test).

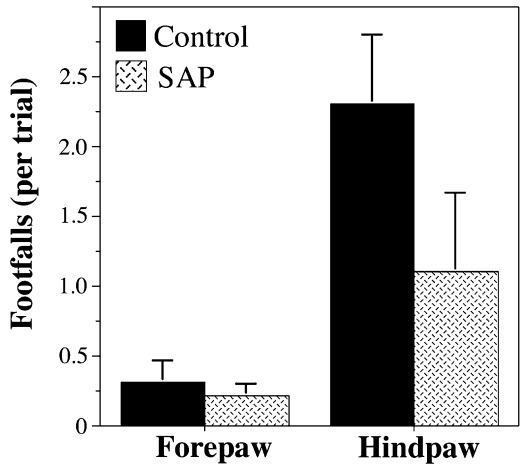

Figure 7. Immunotoxic Lesions of the Basal Forebrain Cholinergic System Do Not Cause Generalized Sensorimotor Deficits

Control and SAP-lesioned (NBM + MS) animals were evaluated on their ability to cross a horizontal ladder, a task previously shown to be sensitive for detecting sensorimotor deficits. Control and SAP-lesioned animals made relatively few errors in crossing the ladder and groups did not differ in the number of forelimb or hindlimb footfalls ($t(10) = -0.59$; $p = 0.57$ for forelimb footfalls and $t(10) = -1.67$; $p = 0.13$ for hindlimb footfalls).

plasticity of cortical representations, regardless of whether the plasticity is induced by normal learning or by peripheral injury/sensory deprivation, and suggest that the underlying mechanisms of plasticity may be similar in all instances. An important point is that, in all cases, the basal forebrain lesion *completely* blocked cortical plasticity, strongly implicating the basal forebrain cholinergic system as an *essential* component in mediating plasticity associated with the reorganization of cortical sensory and motor representations, regardless of the paradigm through which the plasticity is induced.

In the present study, lesions of the basal forebrain cholinergic system not only abolished the expansion of the forepaw representation that normally occurs with learning, but such lesions actually reduced the size of the forepaw representation in trained animals relative to untrained controls. A similar phenomenon was observed within the sensory system following basal forebrain lesions. Juliano and collegues (Juliano et al., 1991) observed that total basal forebrain lesions not only blocked the expected expansion of remaining intact digits following digit amputation in the cat forelimb, but led to a shrinkage of the sensory representation of the remaining digits. Juliano et al. suggested their results indicated that the basal forebrain is needed continuously to maintain normal representations within the cortex.

The significant impairment in skilled motor learning following lesions of the basal forebrain cholinergic system is in contrast to the effects of cholinergic lesions on other forms of learning. In the present study, we demonstrate that the same rats with severe deficits in motor learning have no detectable impairment in learning an associative fear task. Moreover, several previous studies have demonstrated that selective lesions of cholinergic neurons within the NBM produce no, or modest, cognitive deficits in a variety of spatial learning paradigms (Baxter et al., 1995; Baxter and Gallagher, 1996; Chappell et al., 1998; Dornan et al., 1996; Gutierrez et al., 1999; Torres et al., 1994). The apparent specificity of the effect of cholinergic lesions on motor learning, compared to more complex tasks, may be related to the different mechanisms of plasticity underlying the different forms of learning. For example, higher cognitive/associative learning may not utilize cortical reorganization as a primary mechanism for encoding the behavior in the same way that motor learning does. Cortical reorganization has primarily been observed within primary motor and sensory regions; thus, behaviors utilizing these cortical regions for encoding aspects of learning may be more dependent on this cholinergically dependent form of plasticity.

An important observation in this study is that, although lesions of the basal forebrain cholinergic system disrupt cortical plasticity and significantly impair motor skill learning, most lesioned animals do improve in reaching accuracy over time, suggesting that some learning can still occur in the absence of the basal forebrain cholinergic system and cortical map plasticity. One possible mechanism for the residual learning is that remaining cholinergic systems may somehow compensate for the damaged cholinergic neurons. One argument against this possibility is that animals with additional lesions of the medial septum and vertical limb of the diagonal band (in addition to the NBM lesions) did not show a further reduction in learning relative to NBM-lesioned animals. Compensation by cholinergic neurons endogenous to the cortex remains a possibility, albeit unlikely. Another explanation for the residual learning is that motor learning does not require either cortical plasticity or cholinergic modulation, but the cholinergic-dependent plasticity increases the efficiency of learning. Importantly, the basal forebrain cholinergic lesion does not eliminate the forelimb representation but prevents its expansion. Thus, an animal always has some level of cortical control over forelimb movement. The present data suggests that the expansion allocates more cortical resources to controlling the forelimb, thereby enabling the animal to achieve a level of motor refinement necessary to more efficiently execute a skilled movement.

The present data confirm previous reports showing that motor training is associated with an expansion of the cortical representation of the trained limb (Kleim et al., 1998; VandenBerg et al., 2002) and demonstrate that an expansion of the cortical representation of the untrained forelimb also occurs. Moreover, basal forebrain cholinergic lesions abolish cortical plasticity within both hemispheres. The basis for bilateral plasticity following unilateral training is not known. However, previous studies examining morphological plasticity within the primary motor cortex following motor skill learning in rats have demonstrated that increased dendritic complexity occurs bilaterally following unilateral forelimb use (Withers and Greenough, 1989). Anatomical studies have demonstrated extensive callosal projections between hemispheres of the rat primary motor cortex (Donoghue and Parham, 1983), suggesting that information related to plastic changes induced in one hemisphere may be transferred to the contralateral hemisphere. A role for interhemispheric communication has been proposed in bimanual coordination (Gerloff and Andres, 2002), but the nature of plastic changes underlying bilateral coordination of movements remains to be elucidated.

In the current study, the basal forebrain cholinergic system was shown to be essential for inducing cortical plasticity and enabling the flexible allocation of cortical resources presumably required for learning a skilled motor task. The present findings parallel results from previous studies examining attentional performance in rats with selective lesions of corticopetal cholinergic neurons. These studies have indicated that, while aspects of attention are preserved in lesioned rats, these animals are unable to adapt their attention to meet the changing demands of the task (for review see Baxter and Chiba, 1999; McGaughy et al., 2000). The existence of parallel deficits across behavioral domains suggests a more general role for the corticopetal cholinergic system in the induction of cortical plasticity that is essential for the optimization of learning and performance.

Experimental Procedures

A total of 64 adult male F344 rats (starting weight ~325 gm) were used in this study. All procedures and animal care adhered strictly to AAALAC, Society for Neuroscience, and institutional guidelines for experimental animal health, safety, and comfort.

Animal Surgery

All surgical procedures were carried out under ketamine/xylazine/acepromazine anesthesia. Intraparenchymal injections of either 192-IgG-saporin (SAP; Advanced Targeting Systems, San Diego, CA), diluted to a concentration of 0.375 mg/ml in artificial cerebrospinal fluid, or vehicle (artificial cerebrospinal fluid alone) were made using a 0.5 μl Hamilton syringe. The following sites and volumes were injected: for medial septum, site #1 (0.25 μl each side), R/C = +0.8 mm, M/L = ±0.5 mm, D/V = −7.5 mm; site #2 (0.25 μl each side), R/C = +0.0 mm, M/L = ±0.5 mm, D/V = −7.5 mm; for NBM, site #1 (0.3 μl each side), R/C = −1.4 mm, M/L = ±2.5 mm, D/V = −8.0 mm; site #2 (0.2 μl each side), R/C = −2.6 mm, M/L = ±4.0 mm, D/V = −7.0 mm. Injections were made at 0.1 μl per minute and the needle remained in place for 4 min after each injection to allow for diffusion of the injected fluid into the parenchyma. All rats were permitted 7–10 days to recover from the surgery before starting behavioral testing.

Skilled Motor Training

Motor training was carried out using single pellet retrieval boxes. In brief, 31 cm (length) × 21 cm (width) × 19 cm (height) boxes were constructed of clear Plexiglas. In the center of the front wall, a 1.5 cm wide slot was cut from the floor to a height of 5 cm. Outside the slot was a tray with an indentation for holding a single 45 mg sucrose pellet (Research Diets, New Brunswick, NJ). The tray could be adjusted so that the distance from the slot to the pellet could be varied from 1 cm to more than 10 cm.

Beginning 1 week prior to training, all rats were handled on a daily basis and were habituated to the testing chamber. Four days prior to training, rats were weighed and put on food restriction to increase motivation. Weights were monitored daily and standard rat chow was given at the end of each test day to maintain body weight above 80% of the initial weight (2% increase in weight per week was calculated for the course of the study). All rats were initially trained to grasp for food pellets with the tray placed as close to the slot as possible. On the first two testing days, rats were fed reward pellets for orienting to the slot and no reaching was performed. On the third day of training/shaping, rats were encouraged to reach for pellets by adding a small amount of peanut butter to the surface of pellets placed on the tray. Since the peanut butter enhanced pellet retrieval accuracy, by allowing the pellets to stick to the animal's hand (rather than forcing the animal to grasp the pellet), data were not collected on training day 3. On the fourth day of training, the platform was located only 1 cm from the slot and peanut butter was not used during testing. By the fifth training day, the food tray was moved to a distance of 2 cm from the slot and remained at this distance for the duration of the training. Each day, rats were placed into the test box for 10 min or until the rat had made 60 reaches. A "reach" was scored when the rat extended its forelimb through the slot. A "hit" was scored if the rat successfully brought the pellet back to his mouth and consumed it (rats rarely, if ever, did not consume pellets brought back to their mouths, but if the pellet was placed in the mouth it was scored a hit). The time to complete all 60 trials and the limb used by each animal was recorded each session. The order of testing was randomized each day.

Horizontal Ladder Crossing

Overall sensorimotor function was evaluated using a horizontal ladder task as has been described (Soblosky et al., 2001). In brief, 12 rats (6 with bilateral SAP lesions of the NBM and MS + 6 control) were habituated to the apparatus over a period of 2 days and were encouraged to walk across the rungs to reach a platform for a food reward. The ladder consisted of a series of 46 rungs (2 mm thick wire), each 11 cm long, spaced evenly apart (5.2 cm spacing), and was suspended 1 meter above the floor. A digital camera was mounted below the ladder to record all trials for further analysis. Each animal was then required to run seven trials over a period of two nonconsecutive days (three trials one day and four trials the next). A trial was counted when an animal crossed the entire span of the ladder. Forelimb footfalls were scored whenever the animal's wrist slipped below the ladder rungs and hindlimb footfalls were scored whenever the ankle slipped below the ladder rungs.

Fear Conditioning

Learned fear can be acquired very rapidly in a single learning session (LeDoux, 2000; Maren, 2001). Rats were trained on fear conditioning in a single session consisting of a 4 min baseline activity period, followed by 10 paired presentations of a 10 s, 2.9 kHz tone, coterminating with a 1 s, 0.5 mA footshock; intertrial interval was 30 s. Training took place in a plexiglass Habitest unit with a grid shock floor (Coulbourn Instruments, Allentown, PA), located inside a sound attenuating box. Activity was recorded using the body heat-activated Activity Monitor with Graphic State software (Coulbourn Instruments). Ten days following the initial training session, rats were tested in a single session consisting of a 4 min baseline period, followed by 10 tone-only presentations. The testing took place in a context unique from the training context in order to control for contextual learning (LeDoux, 2000).

Electrophysiology

Standard microelectrode stimulation techniques were used to derive maps of the motor cortex both ipsilateral and contralateral to the trained paw (see Nudo et al., 1996, for further details). The side of cortex mapped first was randomly determined without prior knowledge of the animal's paw preference during behavioral training. Animals were initially anesthetized with ketamine hydrochloride (70 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and received supplementary doses of the ketamine/xyalzine mixture as needed. Pulled glass electrodes (input impedance ~0.5 MOhm at 300 Hz), filled with 3 M NaCl and containing a 125 μm chlorided silver wire, were used. Microelectrode penetrations were made at 500 μm intervals at a depth of ~1800 μm (corresponding to cortical layers V-VI). Stimulation consisted of a 30 ms train of 200 μs duration monophasic cathodal pulses delivered at 333 Hz from an electrically isolated, constant current stimulator (Axon Instruments, Union City, CA) under the control of a programmable pulse generator (AMPI, Jerusalem, Israel). Two pulse trains were delivered 1.2 s apart, with additional pulse trains delivered as needed to assess body movements evoked by the stimulation. Evoked movements were examined with the animal maintained in a prone position and the limbs supported in a consistent manner. At each penetration site, the stimulating current was gradually increased until a movement could be detected (threshold current). If no movement could be detected at 400 μA, the site was defined as "nonresponsive." The size of the forelimb representation for each animal was determined by multiplying the number of responsive sites evoking a movement of the forelimb by 0.25 mm^2. In rare circumstances where a site elicited movements of the forepaw and an additional part of the body, the site was still attributed with 0.25 mm^2 toward the total forelimb representation area.

Histology

At the end of the behavioral and/or electrophysiological testing, rats were perfused with 75 ml phosphate buffered saline and 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer. One group of animals, containing five intact and five SAP-lesioned rats, was sacrificed and their brains were placed into Golgi-Cox solution for further Golgi analysis of dendritic spine morphology (unrelated to this study). For these animals it was not possible to verify the extent of immunolesions since the immunostaining protocol was not compatible with the Golgi-Cox fixation. Since comparable immunolesions were confirmed in more than 25 animals in our lab, it was inferred that lesions in the 5 Golgi-Cox processed animals were similar. For all other animals, 40 μm coronal sections were cut on a sliding microtome. A series of sections, 480 microns apart, were processed using a thionin stain. An additional series of sections, 240 μm apart, were processed for AChE using a modified Tago method (Di Patre et al., 1993), and an adjacent series of sections (also 240 μm apart) were processed for the p75 receptor according to previously described methods (Conner et al., 1992). AChE was carried out on free-floating sections. Sections were washed briefly in 0.05 M Tris-maleate buffer (pH = 5.7), incubated for 10 min in Tris-maleate buffer containing 6 μg/ml promethazine, and washed two additional times in Tris-maleate buffer. Sections were incubated for 30 min in a 32.5 mM Tris-maleate buffer solution containing 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferrocynide, and 0.52 mg/ml ace-

tylthiocholine iodide, then rinsed five times in 50 mM Tris-HCl (pH = 7.6). Sections were incubated for 5 min in 50 mM Tris-HCl containing 0.25 mg/ml diaminobenzidine tetrahydrochloride and 3 mg/ml nickel ammonium sulfate. Hydrogen peroxide (0.006% final concentration) was added and sections were allowed to incubate for 2–3 more minutes. The reaction was stopped by washing sections 3–4 times in 50 mM Tris-HCl buffer. Free-floating sections (480 μm apart) were also processed for p75 using the 192-IgG monoclonal antibody (Taniuchi and Johnson, 1985) and parvalbumin using MAB1572 (Chemicon, Temucula, CA). In brief, sections were rinsed for 30 min in 0.1 M Tris-buffered saline (TBS), blocked for 60 min in TBS containing 5% normal horse serum, and incubated for 40 hr (at 4°C) in primary antibody (2.5 μg/ml for the 192 IgG and 1:3000 dilution for MAB1572). Bound antibodies were detected by sequentially incubating sections for 3 hr in 1.5 μg/ml biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA) and for 90 min in an avidin-biotin peroxidase reagent (1:250 dilution ABC Elite, Vector Labs). Sections were rinsed and treated with a solution containing 0.04% diaminobenzidine tetrahydrachloride, 0.06% nickel chloride, and 0.06% hydrogen peroxide in 0.1 M Tris-HCl buffer (pH = 7.4). AChE and p75 stained sections were mounted onto gel-subbed slides, dehydrated in a graded series of alcohols, cleared, and coverslipped.

Unbiased sampling techniques were used to estimate the degree of loss in cholinergic innervation to the sensorimotor cortex following SAP lesions. Three AChE-labeled sections spanning the rostral-caudal extent of the sensorimotor cortex were evaluated from each of six control and six SAP-lesioned animals. The density of cholinergic innervation was estimated by counting the number of AChE-positive fibers crossing the two inclusion boundaries of a 15 × 15 μm counting frame (Conner et al., 2001). A stereology computer program (StereoInvestigator) controlled placement of the counting frame within a prescribed sampling area that included either cortical layers II-III or layers V-VI (Zilles, 1985). The distance between sampling sites was 150 μm for layer II-II and 200 μm for layer V-VI. The average area sampled per animal was 2.7 ± 0.082 mm for layer II-III and 2.07 ± 0.12 mm^2 for layer V-VI.

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